Inheritance and diversification of symbiotic trichonymphid flagellates from a common ancestor of termites and the cockroach *Cryptocercus*

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Cryptocercus cockroaches and lower termites harbour obligate, diverse and unique symbiotic cellulolytic flagellates in their hindgut that are considered critical in the development of social behaviour in their hosts. However, there has been controversy concerning the origin of these symbiotic flagellates. Here, molecular sequences encoding small subunit rRNA and glyceraldehyde-3-phosphate dehydrogenase were identified in the symbiotic flagellates of the order Trichonymphida (phylum Parabasalia) in the gut of *Cryptocercus punctulatus* and compared phylogenetically to the corresponding species in termites. In each of the monophyletic lineages that represent family-level groups in Trichonymphida, the symbionts of *Cryptocercus* were robustly sister to those of termites. Together with the recent evidence for the sister-group relationship of the host insects, this first comprehensive study comparing symbiont molecular phylogeny strongly suggests that a set of symbiotic flagellates representative of extant diversity was already established in an ancestor common to *Cryptocercus* and termites, was vertically transmitted to their offspring, and subsequently became diversified to distinct levels, depending on both the host and the symbiont lineages.

Keywords: *Cryptocercus*; termite; symbiosis; Parabasalia; symbiont diversification; evolution of social behaviour

1. INTRODUCTION

Wood-feeding cockroaches in the genus *Cryptocercus* and phylogenetically basal lower termites depend on gut symbiotic flagellates for cellulose digestion (Inoue *et al.* 2000). This symbiotic relationship is considered a key element in the evolution of social behaviour in the hosts (Nalepa *et al.* 2001) and has ecological significance for the decomposition of plant litter in terrestrial ecosystems (Bignell & Eggleton 2000). The gut flagellates belong to either the phylum Parabasalia or the order Oxymonadida (phylum Preaxostyla; Adl *et al.* 2005). Parabasalia comprises a monophyletic but complex assemblage of diverse species and is traditionally divided into two orders, Hypermastigida and Trichomonadida. Hypermastigids are unique in nature, found only in lower termites and *Cryptocercus*, and play a pivotal role in cellulose digestion. Although these gut symbionts are formidably difficult to culture, culture-independent studies based on molecular sequences have already begun (reviewed in Ohkuma 2008). Such studies on molecular phylogeny of the termite symbionts support multiple origins of the hypermastigids (Hampl *et al.* 2004; Ohkuma *et al.* 2005; Carpenter & Keeling 2007) and, reflecting these molecular studies as well as ultrastructural reconsiderations, the hypermastigids have been recently reclassified into three different orders (Brugerolle & Patterson 2001; Adl *et al.* 2005). Among them, Trichonymphida members (commonly

trichonymphids) are abundant in the gut of *Cryptocercus* as well as many termite species.

Since the elaborative description of the flagellates in the gut of *Cryptocercus* cockroaches (Cleveland *et al.* 1934), there has been controversy over the evolutionary origin of the symbionts. Most notably, in *Proc. R. Soc. B* during the early in 1990s, Thorne (1990, 1991) and Nalepa (1991) debated the relative merits of two hypotheses: that these symbionts had been inherited from a common ancestor of *Cryptocercus* and lower termites or had been secondarily transferred from one taxon to the other. Phylogenetic analyses encompassing both host insects and symbiotic flagellates were necessary to resolve the controversy. Recent rigorous phylogenetic studies on the hosts have confirmed the sister-group relationship between *Cryptocercus* and termites, with this clade embedded within cockroach lineages (Lo *et al.* 2000; Inward *et al.* 2007; Ware *et al.* 2008); this is also supported by the parallel phylogeny of intracellular bacteria (*Blattabacterium* spp.) vertically transmitted via the eggs (Lo *et al.* 2003). These observations imply the inheritance of symbiotic flagellates in their common ancestor. However, no comparative phylogenetic study on the gut flagellates in these hosts has been reported, due to the absence of a comprehensive investigation on *Cryptocercus* symbionts. Although recently the phylogenetic positions of some *Cryptocercus* symbionts have been investigated using molecular data (Heiss & Keeling 2006; Carpenter & Keeling 2007), the evolutionary relationship of these protists with their host insects has not been addressed. Since some trichonymphid genera such as *Trichonympha* and *Eucomonympha* are shared in

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Table 1. Representative flagellate genera found in the gut of *Cryptocercus* cockroaches and termites.

flagellate order or family	*Cryptocercus*	termite
Trichonymphidae	*Trichonympha*	*Trichonympha*
Eucomonymphidae	*Eucomonympha*	*Eucomonympha*
		Pseudotrichonympha
Teranymphidae		*Teranympha*
Hoplonymphidae	*Urinympha*	*Hoplonympha*
	Barbulanympha	
Staurojoeninidae	*Idionympha*	*Staurojoenina*
Spirotrichosomidae	*Leptospiro-nympha*	*Spirotrichosoma*
	Macrospiro-nympha	
Spirotrichonymphida		*Spirotrichonympha*
		Holomastigotoides
Cristamonadida	*Prolophomonas*	*Joenia*
		Devescovina
		Stephanonympha
Oxymonadida	*Saccinobaculus*	*Oxymonas*
		Pyrsonympha

the extant hosts, a possibility of lateral transfer of some restricted symbiont taxa remained. Furthermore, there is significant diversity amounting to at least 16 species in 7 hypermastigid genera in the *Cryptocercus* gut. Termites also harbour hypermastigid diversity comprising nearly 25 genera (Yamin 1979). Table 1 lists representative flagellate genera in *Cryptocercus* and termites. Many of these symbiont genera are specific to either host group, implying their parallel diversification. Nevertheless, the origin of this diversity is crucial in understanding their evolutionary history. The reconstruction of a reliable phylogeny of the symbionts is necessary to address these questions.

In this study, molecular sequences of the parabasalid symbionts, particularly those in Trichonymphida, were investigated in *Cryptocercus punctulatus*, and compared with those from termites, in order to test whether the symbionts have been inherited from an ancestor common to the hosts, and to address the evolutionary origin of the extant diversity of trichonymphids.

2. MATERIAL AND METHODS

The collection of Appalachian *C. punctulatus* and the Japanese termites *Hodotermopsis sjoestedti* and *Reticulitermes speratus*, and their maintenance in the laboratory, were described previously (Noda *et al.* 2006). DNA in the gut microbial community of *C. punctulatus* was extracted, purified and used for polymerase chain reaction (PCR) amplification of small subunit (SSU) rRNA gene with primers for eukaryotes as described previously (Ohkuma *et al.* 2000). The amplification products of expected size for parabasalids (approx. 1.5 kbp), not for oxymonads (above 2.0 kbp), were gel fractionated, purified and cloned into pCR2.1-TOPO vector (Invitrogen). Sixty-three clones were sequenced and sorted as described previously (Ohkuma *et al.* 2000), and only representative sequences were used for phylogenetic inference. The flagellate species in *C. punctulatus* were identified by their morphological characters. The cells of trichonymphid species of typical morphology (*Trichonympha acuta*, *Eucomonympha imla*, *Urinympha talea* and *Barbulanympha* sp.)

were manually isolated under a microscope equipped with a micromanipulator (CellTram and Eppendorf) and used for subsequent PCR and reverse transcription-PCR (RT-PCR) of SSU rRNA gene. The PCR using 5–20 manually isolated cells was performed as described previously (Ohkuma *et al.* 2005). A single cell was usually used for RT-PCR (otherwise 20 cells), with reverse transcription using primer Euk1772H and subsequent PCR with primers Euk18 and Euk1627 (Ohkuma *et al.* 1998, 2000). The gene sequences encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified by RT-PCR from a single isolated cell in each of the total seven flagellate species and analysed as described previously (Ohkuma *et al.* 2007*a*); these species were *T. acuta* and *E. imla* in *C. punctulatus*, two *Trichonympha* spp. and two *Eucomonympha* spp. in *H. sjoestedti*, and *Teranympha mirabilis* in *R. speratus*. The database accession numbers of the DNA sequences determined in this study are AB443588–AB443609.

Fluorescence *in situ* hybridization (FISH) for the identification of trichonymphid species was performed according to Noda *et al.* (2006). The sequence specific probes used in this study were: 5′-TGCGCTCCATGGATACCTG-3′ for Cp20, 5′-TGCTAGATTGCATAGCGATTTT-3′ for Cp38, 5′-ATCCAACCATGCATTGCTGC-3′ for Cp07, 5′-GCTAGGTTGCGTGGTGACAT-3′ for Cp13, 5′-GCTAGATTGCACGGTGAAATT -3′ for Cp26, and 5′-TGCTAGATTTTGTGATGAAATTT-3′ for Cp49. Each of these probes was 5′-labelled with 6-carboxyfluorescein and used for FISH simultaneously with a probe for all eukaryotes (Ohkuma *et al.* 1998) 5′-labelled with Texas-Red.

The new sequences were added to the pre-existing alignments (Ohkuma *et al.* 2005, 2007*a*,*b*) and manually refined by juxtaposing conserved secondary structure in the case of SSU rRNA gene. For the SSU rRNA gene sequences of *Pseudotrichonympha* species, only three among 16 sequences from different termite species were used in the analyses; these 16 sequences were closely related and formed a robust monophyletic group (Noda *et al.* 2007). The maximum-likelihood (ML) tree was inferred with PHYML2.4.4 (http://atgc.lirmm.fr/phyml/) using the general-time reversible model with gamma-distributed rate variation and a proportion of invariable sites. Gamma shape parameter and fraction of invariable sites were estimated from the data. Bootstrap values were obtained from 100 replicates. Bayesian analysis was performed with MRBAYES v. 3.1.2 (http://morphbank.ebc.uu.se/mrbayes/) using the same model as described above. Three hot and one cold Markov chains were run in duplicate, each from a random starting tree for 2 000 000 generations sampled every 1000 generations with burn-in values set at 200 000 generations. GAPDH amino acid sequences were also analysed with PHYML and MRBAYES as described above using the JTT substitution model with gamma distributed rate variation and a proportion of invariable sites. Differences in alternative tree topology were compared by the approximately unbiased (AU) test implemented in CONSEL (http://www.is.titech.ac.jp/~shimo/prog/consel/). The alternative tree topologies were obtained by Bayesian inference under constraint of considered taxa. The sets of GAPDH sequences examined for the monophyletic constraints were as follows: (i) CpT20 and CpT23 of *T. acuta*, *Trichonympha agilis* and *Trichonympha* p. HsT29; (ii) CpT20 and CpT23 of *T. acuta* and *Trichonympha* sp. HsT36; (iii) all the four in the genus *Eucomonympha*; (iv) all the four in the genus *Eucomonympha*
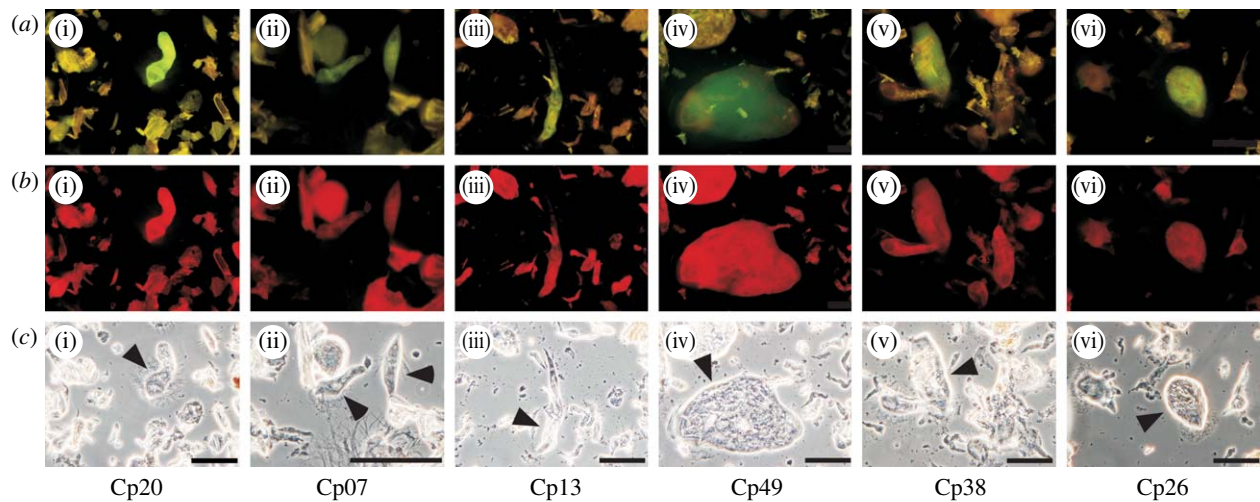
Figure 1. FISH identification of trichonymphid symbionts in the gut of *Cryptocercus*. Six rows correspond to FISH experiments against the gut content of *C. punctulatus* using specific probes for the trichonymphid SSU rRNA gene sequences. The target sequence is shown below images in each row. (*a* (i)–(vi)) Detection of sequence-specific hybridizations (in green), (*b* (i)–(vi)) the control detections of all the eukaryote cells (in red) and (*c* (i)–(vi)) the phase-contrast images of the same microscopic field. The amorphous yellow derives from autofluorescence probably from ingested wood. Arrowheads in the phase-contrast images indicate the cells that gave positive signals for sequence-specific FISH detection. The identified trichonymphid species were as follows: *T. acuta* for Cp20; *Trichonympha* sp. for Cp07; *U. talea* for Cp13; *B. ufalula* for Cp49; and *Barbulanympha* spp. for Cp38 and Cp26. Bars in the phase-contrast images represent 100 μm.

and *T. mirabilis*; and (v) CpT20 and CpT23 of *T. acuta*, and CpE21 and CpE23 of *E. imla* (all the *Cryptocercus* symbionts). The constraints of the sets (i) and (ii) were used for evaluating the nesting of cockroach *Trichonympha* within termite *Trichonympha*. The inference under the set (iv) constraint resulted in the basal position of *P. grassii* in the Eucomonymphidae plus Teranymphidae group.

3. RESULTS

Thirteen representative sequences of parabasalian SSU rRNA gene were obtained from *C. punctulatus*. The FISH experiments using sequence-specific probes (figure 1), and/or the clonal analyses of PCR or RT-PCR products of manually isolated trichonymphid cells, identified the species origins of these sequences; these were *Trichonympha acuta*, *Trichonympha* sp., *Urinympha talea*, *Barbulanympha ufalula*, two *Barbulanympha* spp. and *Eucomonympha imla*. Although the sequence of *E. imla* was identified only from the manually isolated cells, the sequence showed 96 per cent identity to that recently reported for this species (Carpenter & Keeling 2007). These sequences were phylogenetically analysed along with published sequences for trichonymphids in diverse termites (Ohkuma *et al.* 2005 and references therein; Noda *et al.* 2007; see also figure 2 for termite taxa sampled).

In the phylogenetic tree inferred from the SSU rRNA gene sequences (figure 2), four sequences from *Crypto-cercus*, including two identified *Trichonympha* sequences, formed a robust monophyletic lineage. This *Trichonympha* lineage was a sister to *Trichonympha* sequences from five termite genera in evolutionarily diverse positions, although the monophyly of all the *Trichonympha* sequences was only weakly supported (ML bootstrap value/Bayesian posterior probability is 74/46). The sequences of *Barbulanympha* and *Urinympha* (both belong to Hoplonymphidae) formed a robust monophyletic lineage that was sister to *Hoplo-nympha*, the only Hoplonymphidae genus known in termites. *Eucomonympha*, *Pseudotrichonympha* (both belong to Eucomonymphidae) and *Teranympha* (Teranymphidae) formed a monophyletic group; however, the nested position of *E. imla* was dubious. The grouping of Trichonymphida was robustly supported (100/100) in a large phylogenetic tree using a broad sampling of parabasalian taxa (data not shown), in which the root of Trichonymphida located at the node dividing (Trichonymphidae + *Staurojoenina*) and (Hoplonymphidae + Eucomonymphidae + *Teranympha*) when the other parabasalian taxa were treated as out-groups and the monophyletic groups described above were confirmed. In this large tree, the other four sequences from *Cryptocercus* were found to be close relatives of *Hexamastix* spp. from reptiles (Hampl *et al.* 2004) and three unidentified sequences from termites (clones Cbre1, Gf8 and Cd5) (Keeling *et al.* 1998; Ohkuma *et al.* 2000).

The sequences of *Trichonympha* and *Eucomonympha* from *Cryptocercus* were extremely divergent and showed very long branches in the tree, which was in clear contrast to *Trichonympha* in termites and to *Barbulanympha* and *Urinympha* in *Cryptocercus*. These divergent sequences were considered to encode authentic rRNA because near-identical sequences were obtained from RNA by RT-PCR. Base frequency among the trichonymphid sequences was homogeneous (χ^2-test; $p = 0.996$, $\chi^2_{75} = 46.33$). Artificial inference known as long-branch attraction (Felsenstein 1978) was seemingly not the case because step-wise exclusions of these taxa resulted in no substantial change of tree topology with slight increase in support values for some nodes (data not shown).

Since some phylogenetic relationships involving *Tricho-nympha* and *Eucomonympha* in *Cryptocercus* were poorly resolved in the SSU rRNA gene tree, the relationships of these symbionts between *Cryptocercus* and termites were investigated in the more robust analysis using another molecular sequence, GAPDH. The GAPDH sequence has been shown to be useful as a phylogenetic marker in Parabasalia despite the presence of multiple sequences in the genome (Gerbod *et al.* 2004; Ohkuma *et al.* 2007a). The GAPDH gene sequences were identified from
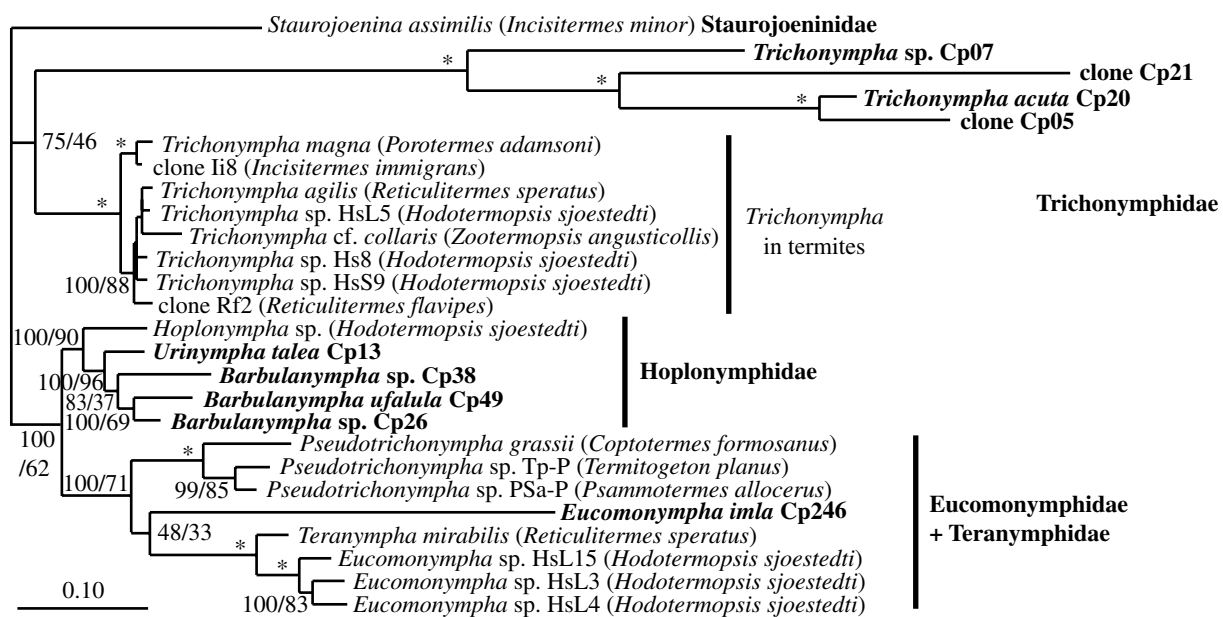
Figure 2. Unrooted ML tree inferred from nuclear SSU rRNA gene sequences of trichonymphids, using 1142 unambiguously aligned positions. The sequences in bold were obtained from trichonymphids in the gut of *C. punctulatus*. The name of host termite species is shown in parentheses after each flagellate taxon. The family-level groups and sequences of termite *Trichonympha* are indicated with vertical bars. The accession numbers of the sequences of the symbionts in termites have been reported in Ohkuma *et al.* (2005) and Noda *et al.* (2007). Bayesian posterior probability and PHYML bootstrap value (divided by slash) are indicated in each node except nodes for branches of termite *Trichonympha*. Asterisks at nodes indicate the full support (100/100). Scale bars indicate 0.10 substitutions per position.

T. acuta and *E. imla* in *Cryptocercus* with RT-PCR using the manually isolated cells. Since the available GAPDH sequences of termite symbionts were still limited, the sequences of *Trichonympha* spp., *Eucomonympha* spp. and *T. mirabilis* were also determined in this study. These taxon samplings covered almost all the genera in Trichonymphidae, Eucomonymphidae and Teranymphidae. Although the available sequences were limited in *Trichonympha*, the samplings were considered to be sufficient because *Trichonympha* species in both *Cryptocercus* and termites formed completely separate groups, each of which was robustly monophyletic in the SSU rRNA gene tree.

The phylogenetic analyses of the GAPDH sequences gave a fully resolved tree (figure 3). The robust monophyletic groupings were inferred in both the *Trichonympha* sequences and the sequences of the Eucomonymphidae plus Teranymphidae members. In each of these monophyletic groups, the sequences of the *Cryptocercus* symbionts showed a sister-group relationship with the sequences from termites. Indeed, the AU tests indicated that the nesting of the *T. acuta* sequences within the *Trichonympha* sequences in termites was rejected ($p < 0.03$). The branching order within the monophyletic group of Eucomonymphidae plus Teranymphidae was significantly supported, although the basal position of *Pseudotrichonympha* in this group was not rejected completely ($p = 0.19$), which was revealed by the series of AU tests (see §2). The monophyletic grouping of all the trichonymphid symbionts in *Cryptocercus* was completely rejected by the AU tests in the dataset of GAPDH as well as SSU rRNA gene (each $p < 0.01$).

4. DISCUSSION
This is the first molecular phylogenetic study comprehensively comparing trichonymphid flagellates between *Cryptocercus* and termites. The results indicate that

Cryptocercus symbionts probably have a sister-group relationship to the corresponding symbionts of termites in each group of Trichonymphida. The nesting of *Cryptocercus* symbionts within clades of corresponding termite symbionts was rejected. Since the host *Cryptocercus* is phylogenetically sister to termites, the results strongly suggest that a set of trichonymphid flagellates that correspond to ancestors of extant Trichonymphidae, Hoplonymphidae and Eucomonymphidae plus Teranymphidae was established in the common ancestor of the hosts and vertically transmitted to offspring. One line of evidence supporting this hypothesis is that species in the genus *Trichonympha*, one of the few genera common to *Cryptocercus* and termites, are clearly differentiated. *Trichonympha* cells in *Cryptocercus* all possess a nuclear sleeve that is absent in termite *Trichonympha* species; they also differ in their capacity for forming cysts (Kirby 1947).

Proctodeal trophallaxis, the direct transfer of hindgut fluids from the rectal pouch of a donor to the mouth of a receiver, assures the vertical transmission of the symbiotic flagellates from generation to generation in both *Cryptocercus* and termites (Nalepa *et al.* 2001). This mode of symbiont transfer is reflected in cospeciation between *Pseudotrichonympha* species and their host termites (Noda *et al.* 2007) and is also suggested by overall congruence of symbiont composition with host termite phylogeny (Kitade 2004). Vertical transmission via proctodeal trophallaxis in a common ancestor of *Cryptocercus* and termites probably originated from intraspecific coprophagous behaviour in aggregations; coprophagy is common in many studied cockroaches and plays a role in the horizontal transfer of their gut microbiota. It is hypothesized that the shift from horizontal to vertical transmission was correlated with the evolution of subsociality from gregarious ancestors, and resulted in increased interdependence of the hosts and their gut fauna (Nalepa *et al.* 2001). It is becoming increasingly
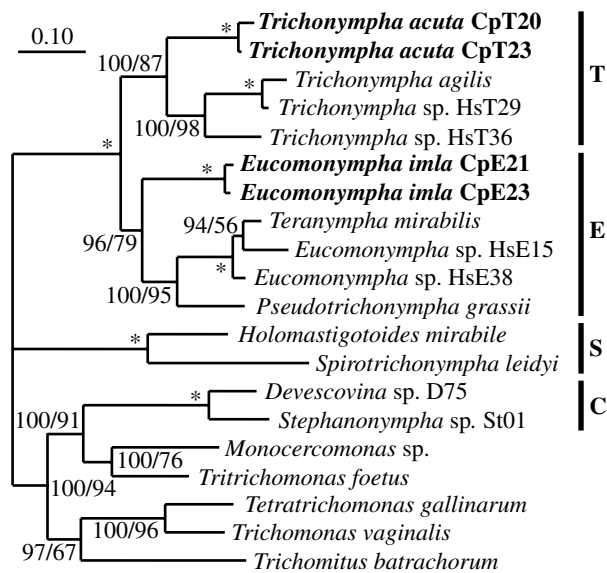
Figure 3. ML tree of GAPDH sequences showing sister relationships of trichonymphid symbionts between *Cryptocercus* and termites. The tree was inferred from 324 amino acid positions aligned unambiguously. The parabasalid sequences outside Trichonymphida were used as out-groups owing to the clear dichotomy between Trichonymphida and the other parabasalids as well as the most likely root position of parabasalids at the node dividing these groups (Ohkuma *et al.* 2007*a,b*). The sequences of trichonymphids in *C. punctulatus* are shown in bold. The family-level groups (T: Trichonymphidae, E: Eucomonymphidae plus Teranymphidae) and the orders Spirotrichonymphida (S) and Cristamonadida (C) are indicated with vertical bars. The accession numbers of the other sequences have been reported in Gerbod *et al.* (2004) and Ohkuma *et al.* (2007*a,b*). Bayesian posterior probability and PHYML bootstrap value (divided by slash) are indicated in each node. Asterisks at nodes indicate full support (100/100). Scale bars indicate 0.10 substitutions per position.

recognized that complexity of social behaviour can be associated with the mode of acquisition of symbionts (Lombardo 2008).

The strict vertical transmission of trichonymphids suggested in this study implies that the set of flagellate species in the common host ancestor represents the origins of present-day diversity of symbionts; the inherited symbionts subsequently diversified within each host lineage. The extent of the species diversification, however, depends on the group of symbiotic flagellates. Species of *Trichonympha* are rich in both *Cryptocercus* and termites. In the case of Hoplonymphidae, *Cryptocercus* stably harbours *U. talea* and at least three species of *Barbulanympha* while the sole genus *Hoplonympha* inhabits only a few termite species. It is likely that species in *Hoplonympha* have been lost in most lineages of termites. The fully resolved GAPDH data showed the basal position of *E. imla* in the Eucomonymphidae plus Teranymphidae group, suggesting that this group of trichonymphid genera may have diversified after the termite lineage separated from the *Cryptocercus* lineage. *E. imla* is the only member in Eucomonymphidae in *Cryptocercus*. By contrast, termites harbour several *Eucomonympha* species, the monospecific genus *Teranympha* and a number of *Pseudotrichonympha* species; each of the first two genera is known to inhabit only a few termite species. As previously shown

(Carpenter & Keeling 2007) and confirmed here, *Eucomonympha* spp. in termites are distantly related to *E. imla* in *Cryptocercus*, and rather closely related to *Teranympha*. Indeed, the monophyly of *Eucomonympha* was rejected by the AU tests with the GAPDH data as well as SSU rRNA gene data (each $p < 0.01$). This indicates that the acquisition of *Eucomonympha* by a lateral transfer between *Cryptocercus* and termite lineages is unlikely. The results also suggest that the morphological characteristics that differentiate *Teranympha* (and probably *Pseudotrichonympha*) from *Eucomonympha* are autapomorphies.

Associations of bacteria with the symbiotic flagellates are a prominent feature in the gut microbial community of termites and play important roles in the efficient usage of cellulose for both host flagellates and termites (Hongoh *et al.* 2008; Inoue *et al.* 2008; Ohkuma 2008). Members of the Hoplonymphidae possess ectosymbiotic bacteria attached to the surface of the flagellate cells and, as previously reported (Noda *et al.* 2006), these ectosymbionts form a monophyletic lineage in the order Bacteroidales. Many species of *Trichonympha* in termites harbour endosymbiotic bacteria belonging to the candidate phylum 'Termite group 1' (Ikeda-Ohtsubo *et al.* 2007; Ohkuma *et al.* 2007*b*; Hongoh *et al.* 2008) and *Pseudotrichonympha* species examined so far harbour endosymbiotic Bacteroidales bacteria (Noda *et al.* 2005, 2007). However, no abundant association of endosymbiotic bacteria was observed in *Trichonympha* spp. and *E. imla* in *Cryptocercus* when they were examined by microscopic observation after DNA staining, although some ectosymbiotic bacteria were present as described previously (Carpenter & Keeling 2007). The endosymbionts were probably acquired only in the flagellate lineages in termite guts after the divergence of the host insects. These observations also support the host lineage-dependent evolution of flagellate symbionts.

It is remarkable that *Cryptocercus* cockroaches retain more diverse flagellate species than any extant termite species. Among molecularly yet-uncharacterized genera in *Cryptocercus*, *Idionympha* (Staurojoeninidae) is probably sister to *Staurojoenina* in termites. *Leptospironympha* and *Macrospironympha* were originally described as belonging to Spirotrichonymphidae (Spirotrichonymphida; Cleveland *et al.* 1934); however, they are now classified into Spirotrichosomidae (Trichonymphida), none of which have been investigated by their molecular sequences. *Prolophomonas* was formerly classified as Lophomonadida (included in the previous Hypermastigida), but now Lophomonadida is reclassified into Cristamonadida (Brugerolle & Patterson 2001). Because molecular phylogeny and traditional classification is often incongruent in Parabasalia (Hampl *et al.* 2004; Ohkuma *et al.* 2005; Noël *et al.* 2007), some uncertainties as to their evolutionary positions remain. Since Spirotrichonymphida and Cristamonadida exclusively comprise symbionts of termites, the yet-uncharacterized genera in *Cryptocercus* discussed above are possible candidates for sister taxa to these orders found in termites. Of course, this possibility is somewhat speculative and unfortunately we failed to detect sequences likely to have derived from these genera in *C. punctulatus*. Future molecular identifications of these are of significant importance for understanding the origin and evolution of these orders. The situation of the oxymonads, which are also unique to *Cryptocercus* and

termites, seems to be similar. The reported SSU rRNA gene sequences of the oxymonads in *Cryptocercus* (Saccinobaculidae) showed a sister-group relationship to Pyrsonymphidae and Oxymonadidae species in termites; however, the resolution of phylogenetic relationships of these three families was poor (Heiss & Keeling 2006). The analysis of protein-encoding genes has already started in oxymonads of *Cryptocercus* (de Koning *et al.* 2008), but studies of those from termites are still limited. Considering that *Cryptocercus* probably harbours the descendants of the original set of symbiotic flagellates that represent their extant diversity, *Cryptocercus* is an important 'model taxon' not only for termite evolution (Nalepa 1988, 1994; Klass *et al.* 2008) but also for the evolution of Parabasalia and Oxymonadida as well.

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